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Method and Nucleic Acids for the Detection of Microorganisms Relevant to Brewing

The invention relates to a method for the detection of microorganisms relevant to brewing, as well as to nucleic acids and combinations thereof which can be used in this method. The invention further relates to the use of the nucleic acids according to the invention or combinations thereof for the detection and/or for the identification and/or characterisation of different genera or species of microorganisms relevant to brewing.

Beer can be regarded as very stable microbiologically, and can only be spoilt by a relatively manageable number of bacteria. In order to discover contamination with these organisms as early as possible, an analytical system which allows rapid detection of the microorganisms in the matrix beer must be used, since countermeasures must be undertaken immediately.

The common feature of all microorganisms harmful to beer is the trace contamination of individual vessels (barrels, bottles) and their slow growth. In particular, microbiological culturing of the anaerobic microorganisms is very difficult. The beer-spoiling bacteria at present known are classed into the following genera: *Lactobacillus, Pediococcus, Pectinatus* and *Megasphaera*. Members of the *Selenomonas* and *Zymophilus* genera have not yet emerged as beer contaminants; however, contamination of beer and their subsequent growth in it cannot be ruled out.

The genus *Lactobacillus* describes Gram positive, non-sporulating, mostly immotile and chain-forming rods, which are long, thin and sometimes curved. Coccoid forms are also sometimes observed. Members of the genus *Lactobacillus* are microaerophilic, and some are anaerobic. They are cytochrome- and catalase-negative, their metabolism is fermentative and they require a complex nutrient medium. The molar G+C content of the DNA is between 32 and 53%.

As well as in beer, *Lactobacilli* are found in dairy and cereal products, in meat and fish products, in water, waste water, wine, fruit and fruit juices, acid-pickled vegetables, sauerkraut, silage and sourdough. Although they are a component of the normal oral, intestinal and vaginal flora of mammals, they are however seldom pathogenic (*Bergeys Manual of Syst. Microbiology*, 1984, p. 1209-1234). In beer, because of their metabolic

products, they lead to clouding and undesired flavour changes. Species relevant to beer spoilage are *Lactobacillus brevis, Lactobacillus lindneri, Lactobacillus casei, Lactobacillus paracasei, Lactobacillus coryniformis* and *Lactobacillus curvatus* (Back, *Brauwelt*, 1980, 120, p. 1562-1569).

The genus *Pediococcus* includes Gram positive, immotile and non-sporulating cocci. They form tetrads or occur as pairs. They are facultative anaerobes, and their oxygen sensitivity differs from species to species. *Pediococci* are cytochrome and catalase-negative and require a complex nutrient medium (*Bergeys Manual of Syst. Microbiology*, 1984, p. 1075-1079). They are used as starter cultures for the production of raw sausage products, they ferment various types of pickled vegetables and lead to the spoilage of foodstuffs (Firnhaber, Baumgart: *Mikrobiologische Untersuchung von Lebensmitteln*, 1993, p. 413-419, 115-117). The genus includes 8 species, and the species *Pediococcus damnosus* and *Pediococcus inopinatus* should be regarded as harmful to beer.

The genus *Pectinatus* includes the species *Pectinatus cerevisiiphilus, Pectinatus frisingiensis* and the strain *Pectinatus sp.* DSM 20764, not further taxonomically classified. All strains have been isolated from spoilt beer (Schleifer et al., *Int. J. of Syst. Bacteriology*, 1990, p. 19-27). These are slightly bent, non-sporulating rod-shaped bacteria. They have comb-like flagella, and are motile. They produce neither catalase nor cytochrome oxidase, and are obligate anaerobes. The molar G+C content is 38-41%. In the genus *Pectinatus*, and also in the genera *Megasphaera, Selenomonas* and *Zymophilus*, the cell wall is more similar to the Gram-positive bacteria than to the Gram-negative bacteria. Although the Gram staining is negative, they are taxonomically classified among the Gram-positive bacteria (Haikara, *The Prokaryotes*, 2nd Edition, Vol. II, 1991, p. 1993-2004).

The genus *Megasphaera* includes the species *Megasphaera elsdenii* and *Megasphaera cerevisiae*. Only *Megasphaera cerevisiae* is relevant to brewing, and is described as a Gram negative, strictly anaerobic, cytochrome- and catalase-negative, immotile and sometimes slightly stretched coccus, which occurs singly, in pairs or in short chains. The mean cell diameter is about 1.4 μm, and the molar G+C content 42.4-44.8%. Main metabolites are sulphur compounds, such as H_2S and volatile fatty acids. In beer,

contamination with *Megasphaera cerevisiae* leads to very marked changes in aroma and taste (Haikara, *The Prokaryotes*, 2nd Edition, Vol. II, 1991, p. 1993-2004).

Species of the genus *Selenomonas* are defined as obligate anaerobes, Gram negative, non-sporulating, slightly curved and motile rods. The molar G+C content is about 48-58% (Schleifer et al., *Int. J. of Syst. Bacteriology*, 1990, p. 19-27). *Selenomonads* are isolated from the stomach and intestinal tract and the dung of mammals. The genus includes 10 species (Hespell et al., *The Prokaryotes*, 2nd Edition, Vol. II, 1991, p. 2005-2013). Only *Selenomonas lacticifex* has been isolated from starter yeast, and is thus relevant to brewing. *Selenomonas lacticifex* has not yet emerged as a beer-spoiling bacterium; however, its growth in beer is possible, and hence it fulfils the definition of a beer-spoiling organism.

The species *Zymophilus paucivorans* and *raffinosivorans* belong to the genus *Zymophilus* as Gram-negative, slightly bent, motile rods, which occur singly, in pairs or in short chains. The molar G+C content is about 38-41% . They are obligate anaerobes and have a fermentative metabolism. Both species are isolated from starter yeasts and brewery wastes; growth in beer has only been observed with *Zymophilus raffinosivorans* (Schleifer et al., *Int. J. of Syst. Bacteriology*, 1990, p. 19-27).

On the basis of comparison of the 16S rRNA gene sequences, all the genera to be tested are classified among the Gram-positive bacteria with low G+C content. The genera *Pediococcus* and *Lactobacillus* are classified into the *Lactobacillaceae* family, and the genera *Pectinatus, Megasphaera, Selenomonas* and *Zymophilus* into the *Sporomusa* group. The *Sporomusa* group is also described as a group of the Gram-positive Eubacteriales with Gram-negative cell wall (Stackebrandt et al., *The Prokaryotes*, 2nd Edition, Vol. II, 1991, p. 25-26, 33).

A classical microbiological determination of the microorganisms described above can require up to 10 days. However, a markedly faster analysis is desirable, as otherwise unnecessary storage costs arise or the beer being tested has already been delivered. For these reasons, several rapid detection methods have already been developed. Thus, for example, organisms harmful to beer can be detected on the basis of their metabolic products (Haikara et al. Microbiology, 1995, 141, p. 1131-1137). Other indirect methods are turbidometry (Haikara et al., *ASBC*, 1990, p. 92-95) and measurement of

the ATP bioluminescence (Miller et al., *J. Inst. Brew.*, 1989, Vol. 95, p. 317-319). Detection by means of antibodies is also rapid and specific (Gares et al., *ASBC*, 1993, p. 158-163; Winnewisser et al., *Int. J. of Bacteriology*, 1995, 45, p. 403-405). With these methods, the disadvantage is that either non-specific parameters are tested or only one species or genus is detected in each case. Also, the equipment and staff cost is high. An overview of rapid methods for the detection of contaminants relevant to brewing is given by Dowhanick (*Cerevisia*, 1995, 20/4, p. 40-49).

The polymerase chain reaction (PCR; Mullis et al., see US 4,683,195, US 4,683,202 and US 4,965,188) is a rapid and effective method of specifically detecting organisms. A range of nucleic acids are known, through the use of which as primers and/or probes the specific detection of microorganisms relevant to brewing is possible. However, a disadvantage is that with the use of these nucleic acid molecules in an amplification or detection reaction, it is always only possible to detect a fraction of all possible microorganisms relevant to brewing. These PCR systems serve for the specific detection in each case only of individual species in an amplification reaction of the genera *Lactobacillus, Pediococcus, Pectinatus* and *Mega-sphaera* (Sakamoto US 5,869,642; Nietupski et al., US 5,705,339 and US 5,484,900; Tsuchia et al., JP 06141899A, JP 06113888A / *ASBC J.*, 1992, p. 64-67 / *ASBC J.*, 1993, p. 40-41; Yasui JP07289295A / *Can. J. Microbiol.*, 1997, 43, p. 157-163, Shimada et al., JP06090793; Alatossava et al. WO97/09448; Doyle et al., *J. of Ind. Microbiology*, 1995, 15, p. 67-70; DiMichele et al., *ASBC J.*, 1993, p. 63-66; Vogeser et al, *Brauwelt*, 1998, 24/25, p. 1060-1063). Further, the methods described for visualisation of the amplification products, such as, for example, agarose gel electrophoresis, present problems, as the carcinogenic and highly toxic ethidium bromide is used for staining the amplification products. These methods can only be automated with difficulty and the assessment of the agarose gels or the identification of the microorganisms on the basis of the length of the amplification products is sometimes not clear.

The problem to be solved by the present invention was, therefore, to provide a method and means which make possible a rapid test of beer and brewing raw materials for contamination with microorganisms, the test being required to detect the whole range of possible beer-contaminating microorganisms.

This problem is solved according to the invention by a process which comprises the following steps:

(a) bringing the sample into contact with a combination of at least two first nucleic acid molecules (primers), which hybridise with a region of a microbial nucleic acid conserved in microorganisms relevant to brewing;

(b) amplification of the microbial nucleic acid or a portion thereof to produce at least one amplification fragment;

(c) bringing the amplification fragments obtained in step (b) into contact with at least one second nucleic acid molecule (probe), which specifically hybridises with at least one amplification fragment that comprises a sequence of the microbial nucleic acid specific for all microorganisms relevant to brewing or for one or several families, genera or species of microorganisms relevant to brewing; and

(d) detection of at least one hybrid nucleic acid which consists of an amplification fragment and a second nucleic acid molecule introduced in step (c),

and by a nucleic acid molecule selected from:

(i) a nucleic acid with a sequence according to SEQ ID NO 1-107 or a fragment thereof at least 10, preferably 15-30, nucleotides long;

(ii) a nucleic acid which specifically hybridises with a nucleic acid according to (i);

(iii) a nucleic acid which is at least 70%, preferably at least 90%, identical with a nucleic acid according to (i) or (ii), or

(iv) a nucleic acid which is complementary to a nucleic acid according to (i) to (iii).

In the sequences according to SEQ ID NO 1-107, nucleotides are abbreviated as follows: G = guanosine, A = adenosine, T = thymidine, C = cytidine, U = uracil, i = inosine. In accordance with IUPAC, mixtures are abbreviated as follows: R = G or A, Y = C or T, K = G or T, W = A or T, S = C or G, M = A or C, B = C, G or T, D = A, G or T, H = A, C or T, V = A, C or G, and N = A, C, G or T.

For the determination of identity (in the sense of complete agreement, corresponding to 100% identity) with nucleic acid sequences according to (iii), partial sequences of a larger polynucleotide are considered. These partial sequences include 10 nucleotides and are identical when all 10 building blocks are identical in the two sequences compared. The nucleotides thymidine and uridine are to be regarded as identical. All possible fragments of a larger polynucleotide can be regarded as partial sequences.

Here 90% identity is present, when in the two sequences to be compared 9 out of 10 or 18 out of 20 nucleotides in one section are identical.

As an example, let us consider two polynucleotides which comprise 20 nucleotides and differ in the 5th element. In a sequence comparison, six 10-nucleotide ones are then found which are identical, and 5 which are not identical, as they differ in one element.

Otherwise, the identity can also be determined by degree, the unit being stated in percent. For determination of the degree of identity, partial sequences are also considered, which as a minimum include the length of the sequence actually used, e.g., as primer, or else 20 nucleotides.

As an example, polynucleotides A with a length of 100 nucleotides and B with a length of 200 nucleotides are compared. From polynucleotide B, a primer with a length of 14 nucleotides is derived. For the determination of the degree of identity, polynucleotide A is compared with the primer over its whole length. If the sequence of the primer occurs in polynucleotide A, but differs in one element, then there is a fragment with a degree of identity of 13/14 → 92.3%.

In the second example, the whole of the aforesaid polynucleotides A and B are compared. In this case, all possible comparison windows of a length of 20 nucleotides are applied, and the degree of identity determined for them. Thus, if nucleotides 50-69 of polynucleotide A and B are identical with the exception of nucleotide No. 55, then for these fragments a degree of identity of 19/20 → 95% is found.

The method according to the invention can be carried out more rapidly than the previous microbiological detection methods, and makes it possible to detect several, preferably all, microorganisms relevant to brewing potentially present in a sample, such as, for example, even *Lactobacillus* species or members of the genera *Selenomonas* or

Zymophilus seldom arising as contaminants, for which hitherto no detection method existed. The detection is comprehensive and indicates all contamination risks in the brewery. By means of the method according to the invention, microorganisms relevant to brewing can be detected both in beer samples and also in raw material samples (barley malt, yeast, hops, water) or samples of intermediate products in beer production (e.g. mash, wort) even when the number of contaminating microorganisms is still low.

In this context, microorganisms relevant to brewing are understood primarily to mean bacteria and in particular the bacteria described above, *Lactobacillus brevis, Lactobacillus lindneri, Lactobacillus casei, Lactobacillus paracasei, Lactobacillus coryniformis, Lacto-bacillus curvatus, Pediococcus damnosus, Pediococcus inopinatus, Pectinatus cerevisii-philus, Pectinatus frisingiensis, Pectinatus sp.* DSM 20764, *Megasphaera cerevisiae, Selenomonas lacticifex, Zymophilus paucivorans* and *Zymophilus raffinosivorans*, and also all microorganisms to be found in beer, which, while they do not belong to the aforesaid species, can nonetheless multiply in beer, for example, rare members of the *Lactobacillaceae* family, such as *Lactobacillus malefermentans, Lactobacillus buchneri, Lactobacillus parabuchneri, Lactobacillus sanfrancisco, Lactobacillus delbrueckii, Leuconostoc mesenteroides, Pediococcus pentosacaeus* and *Lactococcus lactis*.

The microorganisms detectable by the method according to the invention are, thus, not limited to the microorganisms hitherto described as beer contaminants. Rather, the use of the nucleic acid molecules and the method according to the invention offers the possibility of recognising the presence of other microorganisms relevant to brewing, which have not previously been described as beer contaminants. A positive result at the level of higher taxonomic units (e.g. orders, families, genera) combined with a negative result at the level of the lower taxonomic units known to be relevant to brewing (e.g. species, subspecies, strains) indicates a contamination with such a non-typical microorganism relevant to brewing.

In a first step of the method according to the invention, the sample to be tested is brought into contact with a combination of at least two first nucleic acid molecules (primers). These nucleic acid molecules hybridise with a region of a microbial nucleic acid which is conserved in microorganisms relevant to brewing. The hybridisation takes place through pairing of the primer with regions of the microbial nucleic acid which have

an at least partly complementary base sequence. The term "conserved" characterises the evolutionary variability of nucleotide sequences for species of different taxonomic units. If corresponding sequence sections from at least two microorganisms relevant to brewing are compared, the sequence can be regarded as variable or as conserved. Comparison sequences which are at least 95% identical are described as conserved, and those which are less than 95% identical as variable. Thus, a region of a nucleic acid conserved in microorganisms relevant to brewing denotes a region which is at least 95% identical in all microorganisms relevant to brewing (as defined above).

In a preferred embodiment of the present invention, the conserved region occurs in a genome section which contains the bacterial 23S and 5S genes. This region includes the intergenic spacer between the genes for the 23S rRNA and the 5S rRNA and the bounding 23S and 5S rDNA genes, and includes both conserved sequence regions and also hypervariable (i.e., very organism-specific) sequence regions. Prokaryotic ribosomes as a rule contain three distinct nucleic acid components, which are generally known as 5S, 16S and 23S rRNA (ribosomal nucleic acid). The genetic information for these ribonucleic acids (rDNA) is typically arranged in the genome as a tandem. The typical organisation of such a unit is 16S-23S-5S, where the genes are connected to one another by short hypervariable intergenic regions, so-called spacers. The units are present several times in the genome, and the number of operons can vary from species to species. The high conservation of the DNA sequence in certain sections of the ribosomal DNA over the whole bacterial kingdom allows the design of non-specific oligonucleotides even without exact knowledge of the individual DNA sequences of the organisms to be investigated. The sequences according to SEQ ID NO 1-20 according to the invention (Table 1) are sequences of the 23S-5S intergenic spacer of microorganisms relevant to brewing, from which nucleic acid molecules for use in the method according to the invention can be derived.

The combination of at least two first nucleic acid molecules used in the first step of the method according to the invention is selected, such that they are usable as primers in an amplification reaction, i.e., one nucleic acid molecule hybridises onto a first conserved region of the first strand of the target DNA and the other nucleic acid onto a second conserved region of the DNA strand complementary to the first, wherein the desired target region of the DNA is included. Both nucleic acid molecules have a length of at

least 10 bp, preferably 15-30 bp. In a preferred embodiment of the invention, a combination of at least two nucleic acid molecules according to this invention is used. In a particularly preferred embodiment of the invention, a combination is used which includes at least one nucleic acid molecule with a sequence according to one of the SEQ ID NO 40 to 47 (Table 2) and at least one nucleic acid molecule with a sequence according to SEQ ID NO 48-54 or SEQ ID NO 55-59 or SEQ ID NO 60-72 (Table 2).

In a second step of the method according to the invention, the microbial nucleic acid or a portion thereof is amplified, whereby at least one amplification fragment is produced. Amplification is understood to mean the raising of the concentration of a nucleic acid or a portion thereof present in a reaction mixture. Processes used for the amplification of nucleic acids are for example the PCR (US 4,683,195, US 4,683,202 and US 4,965,188), the “self-sustained sequence replication” (EP 329,822), the “transcription-based amplification system” (EP 310,229) and the “β-RNA replicase system” (US 4,956,858). In a preferred embodiment of the present invention, the amplification comprises a polymerase chain reaction (PCR). In a further embodiment of the present invention, the amplification comprises a ligase-chain reaction or an isothermal nucleic acid amplification.

In a third step of the method according to the present invention, the amplification fragments obtained are brought into contact with at least one second nucleic acid molecule (probe). This nucleic acid molecule or these nucleic acid molecules hybridise specifically with at least one amplification fragment thatcomprises a sequence of the microbial nucleic acid which is specific for all microorganisms relevant to brewing or for one or several families, genera or species of microorganisms relevant to brewing, i.e., only occurs in members of these families or genera or in these species.

The double-strand formation of two identical or similar nucleotide fragments (DNA, RNA, PNA) is described as hybridisation. The term specific hybridisation is used when a stable hybrid nucleic acid between the oligonucleotide and the corresponding target DNA of the oligonucleotide exists, but not to other DNA than the target DNA. For the purposes of this invention, the feature “sequence which specifically hybridises with a sequence according to (i)” refers to a sequence, which under stringent conditions, hybridises with the sequence according to (i). For example, the hybridisations can be carried out at 50°C with a hybridisation solution consisting of 2.5 x SSC, 2 x Denhardts solution, 10 mM

Tris, 1 mM EDTA pH 7.5. Suitable washing conditions are for example four times repeated 1-minute washings in 0.1 x SSC to 1.0 x SSC, 2 x Denhardts, 10 mM Tris, 1 mM EDTA, pH 7.5 at 20-50°C.

In a preferred embodiment of the invention, one or several of the nucleic acid molecules according to the invention is used as a second nucleic acid molecule (probe). Consensus probe is understood to mean a nucleic acid molecule which hybridises with highly conserved regions of a microbial nucleic acid and reacts with the amplification products of all microorganisms relevant to brewing. Nucleic acid molecules according to the invention which are usable as consensus probes have a sequence according to one of SEQ ID NO 40 to 72 (Table 2).

For the detection of a specific genus of microorganisms relevant to brewing, a nucleic acid molecule with a sequence according to one of SEQ ID NO 35 to 39 or SEQ ID NO 104 to 107 (Table 2) is preferably used. The genus specificity of a probe is defined as the ability of this probe to hybridise with the DNA of all isolates of as large as possible a group of members of the particular genus to be detected.

Species-specific nucleic acid probes are understood to mean nucleic acid molecules which hybridise with the DNA of all isolates of the particular species to be detected under the same stringency conditions. Species-specific nucleic acid molecules according to the invention with SEQ ID NO 21-22, SEQ ID NO 25-34, SEQ ID NO 73-78, SEQ ID NO 80-85 or SEQ ID NO 87-97 (Table 2) can be used.

The probes SEQ ID NO 23-24, SEQ ID NO 79, SEQ ID NO 86 and SEQ ID NO 98 to 103 are special cases. With the probes according to SEQ ID NO 23 and SEQ ID NO 79, strains of *Lactobacillus casei* and *Lactobacillus paracasei* ssp. *paracasei* can be detected. A probe according to SEQ ID NO 24 allows the detection of two subspecies of *Lactobacillus coryniformis (L. coryniformis* ssp. *coryniformis* and *L. coryniformis* ssp. *torquens*). With the probe SEQ ID NO 86, strains of the species *Pediococcus damnosus, Pediococcus inopinatus* and *Pediococcus parvulus* can be detected. With the use of these probes, other microorganisms relevant to brewing are not detected. Likewise, with the probes SEQ ID NO 98 to 103, all species of the *Lactobacillaceae* family relevant to brewing to be detected are detected, and other species and genera relevant to brewing are discriminated against.

In the last step of the method according to the invention, the detection of at least one hybrid nucleic acid which consists of an amplification fragment and a second nucleic acid molecule introduced in the preceding step takes place.

Preferably, first nucleic acid molecules (primers) and/or second nucleic acid molecules (probes) are at least 10 nucleotides, preferably 15-30 nucleotides long. In one embodiment of the present invention, the first and/or the second nucleic acid molecules are modified in that up to 20% of the nucleotides in 10 consecutive nucleotides, in particular 1 or 2 nucleotides of a block of 10 are replaced by nucleotides which do not occur naturally in bacteria.

The method according to the invention preferably includes the so-called consensus PCR. In this method, multiplication of the microbial nucleic acid or a portion thereof, and subsequent detection of these molecules by hybridisation with labelled specific probes take place. In the consensus PCR, nucleic acid molecules are used which make it possible to obtain an amplification product from several or, indeed, all of the relevant strains, subspecies, species or genera. The amplification does not lead to a differentiation of the microorganisms. The specificity of the detection is achieved through the subsequent hybridisation reaction with specific probes. In this way, microorganisms relevant to brewing can be simultaneously detected in a simple combination of amplification and detection reaction.

This kind of amplification and detection makes it possible to automate the detection reaction, so that a high sample throughput becomes possible. For example, a PCR-ELISA detection procedure can be used, in which the respective probes are bound in different wells of a microtitre plate, in which the hybridisation and the detection of the labelled amplification products then occurs. The detection can also be effected by the use of a microarray, on which several probes are immobilised, as a result of which the detection reaction can be carried out quickly and at no great cost.

In a preferred embodiment of the invention, the second nucleic acid molecule (probe) is modified or labelled in such a way that it can produce a detectable signal. The modification or labelling is selected from (i) radioactive groups, (ii) coloured groups, (iii) fluorescent groups, (iv) groups for immobilisation on a solid phase and (v) groups

which permit an indirect or direct reaction, especially with the aid of antibodies, antigens, enzymes and/or substrates with affinity to enzymes or enzyme complexes.

For the purposes of this invention, labelling indicates directly or indirectly detectable groups or groups for immobilisation on a solid phase, which are attached to the nucleic acid molecule. Directly detectable are metal atoms, radioactive, coloured or fluorescent groups. Indirectly detectable are immunologically or enzymatically detectable groups, for example, antigens and antibodies, haptens or enzymes or enzymatically active parts of enzymes. These indirect groups are detected in subsequent reactions. Preferred are haptens which are coupled to an oligonucleotide and which are detected in a subsequent antibody reaction.

The nucleic acid molecules according to the invention can be used for the detection and/or for the identification and/or characterisation of bacteria relevant to brewing. The primers and/or probes described herein can also be used in the detection of the described microorganisms in drinks other than beer, in other samples from the brewing sector, such as for example in raw materials, starter yeast, environmental samples, in other foodstuff samples or in clinical samples, etc.

Examples:

Example 1: Determination of the DNA target sequence of the bacteria harmful to beer and closely related species

By sequence comparison of known 23S rDNA and 5S rDNA sequences (GenBank Sequence Database of the National Center of Biotechnology Information: NCBI), conserved gene regions were identified, which serve as hybridisation sites for the primers used for the sequencing. From pure cultures of the bacteria listed in Table 1, genomic DNA was isolated by known standard methods. With primers which hybridise in highly conserved regions, amplification products of all bacteria to be detected were obtained in a PCR. The following primers were used for the amplification and the subsequent sequencing:

Primer 1 = SEQ ID NO 47:
5'-AAG TGC TGA AAG CAT CTA AG-3'

Primer 2 = SEQ ID NO 55:

5'-GGC RRY GTC TAY TYT CSC-3'

Composition of the PCR:

Genomic DNA (10 – 100 ng)	1.00 µl	
H_2O	16.85 µl	
Buffer (10 x)	2.50 µl	1 x
dNTP (10 mM)	0.50 µl	200 µM
Primer 1 = Seq ID NO 48 (5 µM)	1.50 µl	0.30 µM
Primer 2 = Seq ID NO 49 (5 µM)	1.50 µl	0.30 µM
$MgCl_2$ (50 mM)	1.00 µl	2.00 mM
Taq-polymerase (5 U/µl)	0.15 µl	0.03 U/µl
Σ	25.00 µl	

Temperature profile:

5 mins	95°C	
30 secs	95°C	
30 secs	50°C	x 38
30 secs	72°C	
5 mins	72°C	

These amplification products were purified via an agarose gel and by a subsequent treatment with the QIAquick PCR Gel Extraction Kit (Quiagen Co.) and sequenced in the Long Read Sequencer Model 4000L (LI-COR Co.) with the aforesaid primers, which are provided with an IRD-800 label. The resulting sequences of the 23S/5S rDNA spacer regions of the bacteria relevant to brewing and the phylogenetically closely related species were compared with one another and sequence regions identified which:

1.) are to be found in all species of the particular genus to be detected and at the same time differ from those of other genera or species,

2.) are only to be found in the particular species to be detected, but differ from other bacteria to be detected and not to be detected.

In the sequence regions described under 1.), hybridisation sites of genus-specific oligonucleo-tides were defined, and in the sequence regions described under 2.), the binding sites of species-specific oligonucleotides were defined.

Example 2: Detection of Bacteria Harmful to Beer by the Polymerase Chain Reaction

I. Amplification

Genomic DNA was isolated from pure cultures of the bacteria listed in Table 1 by known standard methods. Decimal dilutions from 1 fg/µl to 1 pg/µl of these preparations were then used in a PCR with the following composition:

Primer 3 = SEQ ID NO 46:
5'-AAG GGC CAT CRC TCA ACG G -3'

Primer 4 = SEQ ID NO 48:
5'-TGT GTT CGi iAT GGG AAC AGG TG -3'

Genomic DNA	1.00 µl	4.00 µl	
H_2O	16.60 µl	66.40 µl	
Buffer (10 x)	2.50 µl	10.00 µl	1 x
dNTP (10 mM)	0.50 µl	2.00 µl	0.20 mM
Primer 3 = Seq ID NO 21 (5 µM)	1.50 µl	6.00 µl	0.30 mM
Primer 4 = Seq ID NO 22 (5 µM) digoxigenin labelled	1.50 µl	6.00 µl	0.30 mM
DMSO (100%)	0.25 µl	1.00 µl	1.00 %
$MgCl_2$ (50 mM)	1.00 µl	4.00 µl	2.00 mM
Taq-polymerase (5 U/µl)	0.15 µl	0.60 µl	0.03 U/µl
Σ	25.00 µl	100.00 µl	

The PCR was performed under the following conditions in the Mastercycler® (Eppendorf Co.) according to the following temperature profile:

5 mins	95°C	
30 secs	95°C	
45 secs	55°C	x 38
90 secs	72°C	
5 mins	72°C	

Primer 3 (SEQ ID NO 46) was determined by sequence comparison of known 23S rDNA sequences (GenBank Sequence Database of NCBI). It hybridises onto highly conserved sequence sections in the 23S rDNA gene region. The binding site lies outside the region sequenced with the primers SEQ ID NO 48 and 49.

Primer 4 (SEQ ID NO 48) was determined on the basis of our own sequence data. The hybridisation site of primer 2 lies adjacent to the intergenic 23S/5S spacer in the 5S rDNA region.

II. Detection by PCR-ELISA

The detection is effected by PCR-ELISA. For this, per probe used, 5µl of amplification product are treated with 5 µl of denaturation buffer (125 mM NaOH, 20 mM EDTA, pH 14) and incubated for 15 mins at room temperature. Each time, 2 pmoles of the particular biotinylated probe are pipetted into 100 µl of hybridisation buffer (2.5 x SSC, 2 x Denhardts solution, 10 mM Tris, 1 mM EDTA, pH 7.5) and transferred to the wells of a microtitre plate coated with streptavidin and preincubated at the hybridisation temperature of 50°C. After the denaturation, the denaturation mixture is pipetted into the hybridisation mixture. Next the mixture is incubated for 30 minutes at hybridisation temperature. If the hybridisation is complete, the hybridisation mixture is removed and the plate washed 4x with 200 µl of wash buffer 1 (WB1: 0.1 x SSC, 2 x Denhardts, 10 mM Tris, 1 mM EDTA, pH 7.6) for 1 min. each time at hybridisation temperature. Next, 100 µl of a solution of a horseradish peroxidase conjugated anti-digoxigenin antibody diluted according to the manufacturer's instructions is added (Boehringer Mannheim). The conjugate is diluted in wash buffer 2 (WB2: 100 mM Tris, 150 mM NaCl, 0.05% Tween 20, 0.5% blocking reagent, 100 µg/ml herring sperm, pH 7.6). Next, the antibody incubation is performed at 37°C for 30 mins. After this, the plate is washed four times with 200 µl of WB2 (at room temperature). After the washing, 100 µl of POD

substrate (Boehringer Mannheim) are added and the mixture incubated for 20 mins at RT. Next the colour reaction is stopped with 100 µl of 0.5M H_2SO_4 and estimated at 450 nm.

III. <u>Assessment</u>

According to the detection protocol described above, the detection was performed for all bacteria and bacteria groups investigated, using the corresponding genus- and species-specific probes. Genus-specific probes are SEQ ID NO 35 for *Pediococcus*, SEQ ID NO 36 for *Pectinatus*, SEQ ID NO 37 for *Megasphaera*, SEQ ID NO 38 for *Selenomonas* and SEQ ID NO 39 for *Zymophilus*. Species-specific probes are SEQ ID NO 21 for *Lactobacillus brevis*, SEQ ID NO 22 for *Lactobacillus lindneri*, SEQ ID NO 23 for *Lactobacillus casei + paracasei*, SEQ ID NO 24 for *Lactobacillus coryniformis*, SEQ ID NO 25 for *Lactobacillus curvatus*, SEQ ID NO 26 for *Pediococcus damnosus*, SEQ ID NO 27 for *Pediococcus inopinatus*, SEQ ID NO 28 for *Pectinatus cervisiiphilus*, SEQ ID NO 29 for *Pectinatus frisingiensis*, SEQ ID NO 30 for *Pectinatus* sp. DSM20764, SEQ ID NO 31 for *Megasphaera cerevisiae*, SEQ ID NO 32 for *Selenomonas lacticifex*, SEQ ID NO 33 for *Zymophilus paucivorans* and SEQ ID NO 34 for *Zymophilus raffinosivorans.*

As controls, the consensus probes SEQ ID NO 40 and 41 were used, which hybridise with the amplification products of all the species to be detected. Further possible binding sites for consensus probes are SEQ ID NO 42-45. The probes of SEQ ID NO 40 to 45 were determined by sequence comparison of known 23S rDNA and 5S rDNA sequences (GenBank Sequence Database, NCBI).

If the extinction measured for a 1 fg quantity of genomic DNA used in the PCR was greater than 1, the result was assessed as positive. The results of the PCR-ELISA are presented in Table 3.

Table 1

SEQ ID NO	Source: Genus	Source: Species	Source: Strain	Description	Sequence		
1	Lactobacillus	brevis	DSM 20054	23S-spacer-5S	5'-	TATATGGAAG TAAGACCCCT GAGAGATGAT CAGGTAGATA GGCTGGAAGT AGCAGCGCCG TGAGGCGTGG AGCGGACCAG TACTAATCGG TCGAGGACTT AACCAAGTCA ACAACGTAGT TGTTTCGAGA ATAATTGAAT AATATCTAGT TTTGAGGGAA GAAGTTCTCT TATAGTGTGG TGGCGATAGC CTGAAGGATA CACCTGTTCC CATGCCGAAC ACAGAAGTTA AGCTTCAGCA CGCCGATAGT AGTTGGGGGA TCGCCCC-3'	50 100 150 200 250
2	Lactobacillus	lindneri	DSM 20690	23S-spacer-5S	5'-	CCATTCCTAT ATGGAAGTAA GACTCCTGAA AGATGATCAG GTCGATAGGT TAGAAGTGGA AGCATAGTGA TATGTGAAGC GGACTAATAC TAATCAGTCG AGGACTTAAC CAAGGAAGAC ACAGGGTTAA ATCAAAGTTG AACAGAGAAG ATATTATCTA GTTTTGAGAG AACGAAGTTC GCTCAGGCTT ATGAAAAATA AGCATAGTGT GGTGGCGATA GCCTGAAGGA TACACCTGTT CCCATGCCGA ACACAGAAGT TAAGCTTCAG CACGCCAAAA GTAGTTGGGG GATCGCCCCC TGCGAGGATA GGACGATGGT CATAGC-3'	50 100 150 200 250 300
3	Lactobacillus	casei	DSM 20011	23S-spacer-5S	5'-	CCATTCCTAT ATGGAAGTAA GACCCCTGAG AGATGATCAG GTAGATAGGC TGGAAGTGGA AGTGCAGCGA TGCATGGAGC GGACCAGTAC TAATCGGTCG AGGACTTAAC CAAGTAGAGC GTGAGCAGGA GCGCTTAGAA ACCGGAGCAT AAGCGGGCCT GAGTTCGTTG GCCGGGTTTT GGCCAATGGA TTCAGGGTTC TTATGTGGAG GTTTCTGCGA CTGCGAACGC GTTTCGATGA AATACACTGG TTCCCGACAA CACAAAAACA ACAATGATAG CCAGTTTTGA GAGCGCAAAG TTCTCATAAG TGTGGTGGCG ATAGCAAGAA GGATACACCT GTTCCCATGC C-3'	50 100 150 200 250 300 350
4	Lactobacillus	paracasei ssp. paracasei	DSM 20008	23S-spacer-5S operon 1	5'-	CCATTCCTAT ATGGAAGTAA GACCCCTGAG AGATGATCAG GTAGATAGGC TGGAAGTGGA AGTGCAGCGA TGCATGGAGC GGACCAGTAC TAATCGGTCG AGGACTTAAC CAAGTAAGAG TGTGAGCAGG AGCGGTTAGA AACCGGAGCA TAAGCGGGCC TGAGCGTGAT GGCCGGGCTT TGGCCATTGC GGTCAGGGTC CTTATGTGCA GGTTTCTGCG ACTGCGAACA CGTTTCGATG ACAAGTACGT TAAGTTCAAG GCAGCAATTA AACAATGATA GCTAGTTTTG AGAGCGCAAA GTTCTCATAA GTGTGGTGGC GATAGCAAGA AGGATACACC TGTTCCCATG CCGAACACAG AAGTTAAGCT TCTTCACGCC GAGAGTAGTT GGTGGGAAAC TGCCTGCGAG GATA-3'	50 100 150 200 250 300 350 400

SEQ ID NO	Source			Description	Sequence		
5	Lactobacillus	paracasei ssp. paracasei	DSM 20008	23S-spacer-5S operon 2	5'-	CCATTCCTAT ATGGAAGTAA GACCCCTGAG AGATGATCAG GTAGATAGGC TGGAAGTGGA AGTGCAGCGA TGCATGGAGC GGACCAGTAC TAATCGGTCG AGGACTTAAC CAAGTAAGCG TGCAAGCAGG AGCAGGTTTC TGCGACTGCG AACACATTTC GATGACAAGT ACGTTAAGTT CAAGGCAGCA ATTAAACGAT GATAGCCAGT TTTGAGAGCG CAAAGTTCTC ATAAGTGTGG TGGCGATAGC AAGAAGGATA CACCTGTTCC CATGCCGAAC ACAGAAGTTA AGCTTCTTCA CGCCGAGAGT AGTTGGTGGG AAACTGCCTG CGAGGATA-3'	50 100 150 200 250 300
6	Lactobacillus	coryniformis ssp. coryniformis	DSM 20001	23S-spacer-5S	5'-	CTCGAGTTGA GATTTCCCAT TCCTTTATGG AAGTAAGACC CCTGAGAGAT GATCAGGTAG ATAGGTTGGA AGTGGACGTG CCGTGAGGCA TGGAGCGGAC CAATACTAAT CGGTCGAGGA CTTAACCAAG TAGCATGTAC GTAGTGTTAG TTTAAGGGCA AAGAAATGAA TATCCAGTTT TGAGAGCGCA ACGTTCTCAG AAAGTGGTGT GGTGGCGATA GCAAGAAGGA TACACCTGTT CCCATGTCGA ACACAGAAGT TAAGCTTCTT AGCGCCGAGA GTAGTTGGGG GAGCACCCCC TGCGAGGATA GGACGAT-3'	50 100 150 200 250 300
7	Lactobacillus	coryniformis ssp. torquens	DSM 20004	23S-spacer-5S	5'-	CTCGAGATGA GATTTCCCAT TCCTTTATGG AAGTAAGACC CCTGAGAGAT GATCAGGTAG ATAGGTTGGA AGTGGACGTG CCGTGAGGCA TGGAGCGGAC CAATACTAAT CGGTCGAGGA CTTAACCAAG TAGCATGTAC GTGGTGTTAG TTTAAGGGCA AAGAAATGAA TATCCAGTTT TGAGAGCGCA ACGTTCTCAG AAAGTGGTGT GGTGGCGATA GCAAGAAGGA TACACCTGTT CCCATGTCGA ACACAGAAGT TAAGCTTCTT AGCGCCGAGA GTAGTTGGGG GAGCACCCCC TGCGAGGATA GGACGAT-3'	50 100 150 200 250 300
8	Lactobacillus	curvatus	DSM 20019	23S-spacer-5S	5'-	ACGCCTCGAG ATGAGATTTC CCATTCCTTT ATGGAAGTAA GACCCCTGAA AGATGATCAG GTAGATAGGC TAGGAGTGGA AGTACAGCGA TGTATGGAGC GGACTAGTAC TAATCGGTCG AGGACTTAAC CAAAGGTGCA ATGTTAGGCT TTTGAAATGA AATATTACTT ATTATGCAGT TTTGAGAGAA CGAAGTTCTT CTCAGTGCGC AAGCACAAAA TAGTGTGGTG GCGATAGCAA GAAGGATACA CCTGTTCCCA TGTCGAACAC AGAAGTTAAG CTTCTTAGCG CCGATAGTAG TTGGTGGGAA ACTACCTGCG AGGATAGGAC GATGGT-3'	50 100 150 200 250 300
9	Pediococcus	damnosus	DSM 20331	23S-spacer-5S	5'-	GATGAGATTT CCCATTCCAT TTATGGAAGT AAGACCCCTG AGAGATGATC AGGTAGATAG GTTGGGAGTG GAAGTGTAGT GATACATGGA GCGGACCAAT ACTAATCGGT CGAGGACTTA ACCACAAAGT GGTGTTCTCA AGAGAAGGAT TCGATATTAT TTAGTTTTGA GAGAATAAAT TTCTTTCACA CGAGCCGCGT AAGTGGATCG GAGAAGTGTG GTGACGATAG TGAGAAGGAT ACACCTGTTC CCATGTCGAA CACAGAAGTT AAGCTTCTTA ACGCCGAGAG TAGTTGGGGG ATCGCTCCCT GCGAGGATAG GACGATGGTC AATAG-3'	50 100 150 200 250 300

SEQ ID NO	Source			Description		Sequence
10	Pediococcus	inopinatus	DSM 20285	23S-spacer-5S	5'-	AGATGAGATT TCCCATTCCA TTTATGGAAG TAAGACCCCT GAGAGATGAT 50 CAGGTAGATA GGTTGGGAGT GGAAGTGTAG TGATACATGG AGCGGACCAA 100 TACTAATCGG TCGAGGACTT AACCACAAAG TGGTGTTCTC AAAGAGAAGA 150 TTTCGATATT ATTTAGTTTT GAGAGAATAA ATTTCTTTCA CACGAGCCGC 200 GGAAGTGGAT CGGAGAAGTG TGGTGACGAT AGTGAGAAGG ATACACCTGT 250 TCCCATGTCG AACACAGAAG TTAAGCTTCT TAACGCCGAG AGTAGTTGGG 300 GGATCGCTCC CTGCGAGGAT AGGACG-3'
11	Pectinatus	cerevisiiphilus	DSM 20467	23S-spacer-5S	5'-	AAGTGCTGAA AGCATCTAAG CGTGAAACCT GCCTTAAGAT GAGGTTTCCC 50 AGAGCCGTAA GGCTTGGAAG GCACCTTGAA TAAGACGAGG TAGATAGGCC 100 GGGAGTAGAA GTACAGTAAT GTACGAAGCG GACTGGTACT AATAAGCCGA 150 GAGCTTAACT TAAAATCATC GAAAAAAATG TTTGGTCTGA GATTTCTTCT 200 GTGAAGTTTT GAGTGTGCAA GACACTCTGG TTGAAGGGCA GGGAACGTGA 250 GAGCGTAAAA CTGCGGACTT TGGCTCAAAG AGTTAAAGCA TCTGGTGACG 300 ATACCTGGAT GGATCCACCT GTTCCCATTC CGAACACAGT AGTTAAGCAT 350 CCACAGGCTG AAGGTACTTG GGGGGCGACC CCCTGGGAAA ATAGGACACT 400 GCC-3'
12	Pectinatus	frisingensis	DSM 6306	23S-spacer-5S	5'-	AAGTGCTGAA AGCATCTAAG CGTGAAACCA GCTTTAAGAT GAGGTTTCCC 50 AGAACGCAAG TTTGGAAGGC ACCTTGAAGA AGACGAGGTA GATAGGCCGG 100 GAGTGGAAGT ATGGTGACAT ATGAAGCGGA CTGGTACTAA TAAGCCGAGA 150 GCTTAACTTG ATTTCATCAA AAAAGAGAAA TGTTTGGTCA GAGATTTTCT 200 TCTGTGAAGT TTTGAGTGTG CAAGAACACT CGAGAGTATA TAGGTAAAGG 250 AAAAGCAGCA GATAAGTTTC CTGGTTACTG TATATACCGG CTGAGGTGCT 300 GAGGCACTGA AGGCCAGAAC ATCTGGTGGC GATACCTGGA TGGATCCACC 350 TGTTCCCATT CCGAACACAG TAGTTAAGCA TCCACAGGCC GAAGGTACTT 400 GGGGGGCAGC CCCCTGCGAA AATAGGACAC CGCC-3' 450

SEQ ID NO	Source			Description	Sequence		
13	Pectinatus	sp.	DSM 20764	23S-spacer-5S operon 1	5'-	AAGTGCTGAA AGCATCTAAG CGTGAAACCT GCCTTAAGAT GAGGTTTCCC AGAGCCGTAA GGCTTGGAAG GCACCTTGAA GATGACGAGG TAGATAGGCC GGGAGTAGAA GTATGGTGAC ATACGAAGCG GACTGGTACT AATAAGCCGA GAGCTTAACT TAATTTCATC TATAAATGTT TGGTCCTGAT TTCTTCTGTG AAGTTTTGAG TGTGCAAGAT CACTCATGAA AGTATATAGG TAAAGGGAAA GCAGCAGATT AGTTCCTGGT TTACTTTATA TATGAGCACT AAGGTGCAGA AAAGAACGTT TGAGGAAACG CGGCGTTCGT AAACTCACTT TGCGTGCTGA TTATCTCAAT GCTAAAGCAT TAAGATAATT TTAGAGGAAA CGCGCGTTCA CTAGCGTTCA CTCTGCGTAC TTTATTTCTA AGTGCTGAAG CACTAAGAAG GGCAAGGAAA CGCGTCGTTC GCGATGCTCA CTTTGCGTAC TTCATCTCTA GACTGCTAAA GCAGTAAGAT CTGAAGCATC TGGTGGCGAT ACCTGGATGG ATCCACCTGT TCCCATTCCG AACACAGTAG TTAAGCATCC ACAGGCCGAA GGTACTTGGG GGGCAGCCCC CTGCGAGAGT AGGACATCGC C-3'	50 100 150 200 250 300 350 400 450 500 550 600
14	Pectinatus	sp.	DSM 20764	23S-spacer-5S operon 2	5'-	AAGTGCTGAA AGCATCTAAG CGTGAAACCT GCCTTAAGAT GAGGTTTCCC AGAGCCGTAA GGCTTGGAAG GCACCTTGAA GATGACGAGG TAGATAGGCC GGGAGTAGAA GTATGGTGAC ATACGAAGCG GACTGGTACT AATAAGCCGA GAGCTTAACT TAATTTCATC TATAAATGTT TGGTCCTGAT TTCTTCTGTG AAGTTTTGAG TGTGCAAGAT CACTCATGAA AGTATATAGG TAAAGGGGAA GCAGATTAGT TCCTGGTTTA CTTTATATAT GAGCACTAAG GTGCAGAAAA GAACGTCTAA GGAAACGCGG CGTTCGTAGG CTCACTCTGC GTACTTCATC TCTAGACTGC TAAAGCAGTA AGATCTGAAG CATCTGGTGG CGATACCTGG ATGGATCCAC CTGTTCCCAT TCCGAACACA GTAGTTAAGC ATCCACAGGC CGAAGGTACT TGGGGGGCAG CCCCCTGCGA AAGTAGGACA CCGCC-3'	50 100 150 200 250 300 350 400 450
15	Megasphaera	cerevisiae	DSM 20462	23S-spacer-5S operon 1	5'-	GCATCTAAGC GTGAAACCAG CCTAGAGATG AGGTTTCTCA TTACGAAAGT AAGTAAGGTC CCATGAAGAC GACATGGTAG ATAGGCCGGG AGTGGACGTA CAGTAATGTA TGGAGCGGAC CGGTACTAAT AGACCGAGGA CTTGACTTAA GCAGGGAACC CATTTTAAAG AAGCGAAGCG ACGCATAAAA TGGAGTGAGT CGCTTATACC GAATCGCAGA TTCGGTAAAG CAGCGGAGAA TACCAATGCA GCGGCAACAC CAGTTAGCAT AAACTAAGCG GATTCGGAGT GGGTGAGGGA GTTTCGTAGC AGCGTAGGCT AACCCAACCA CCGCTTTCGA AGAAGGCGAA TGGTTTGAAA AAGAGTACAT GCGAAGAAAC GACGAAAGAC TCACAACCAA AACATACAAA CTAAGTAGAT GACATTAGAG TCACACCGAT TGTTAAGATC CGAAATACTT TTCGATGTAG TTGTCAGGAT ACGAATCCTG AAACGAATTC AGTGGTGATG GCTGCAGGGA TCCACCTGTT CCCATACCGA ACACAG-3'	50 100 150 200 250 300 350 400 450 500

SEQ ID NO	Source			Description	Sequence		
16	Megasphaera	cerevisiae	DSM 20462	23S-spacer-5S operon 2	5'-	GCATCTAACC GTGAAACCAG CCTAGAGATG AGGTTTCTCA TTACGAAAGT AAGTAAGGTC CCATGAAGAC GACATGGTAG ATAGGCCGGG AGTGGACGTA CAGTAATGTA TGGAGCGGAC CGGTACTAAT AGACCGAGGA CTTGACTTAA GCAAAGAAGC AATAGAAAGA ACCATGTAGA TGGTGTAAGA GTTAGACGGG TAGTTAAGGT CCGAAATACT TTTCGATGTA GTTGTCAGGA TACGAATCCT GAAACGAATT CAGTGGTGAT GGCTGCAGGG ACCACCTGTT CCCATACCGA ACACAG-3'	50 100 150 200 250 300
17	Selenomonas	lacticifex	DSM 20757	23S-spacer-5S operon 1	5'-	AAGTGCTGAA AGCATCTAGG CGTGAAGCCT GTCCCGAGAT GAAGTATCTC ATGGAGTAAT CCAGTAAGAT TCCTTGAAGA AGACAAGGTA GATAGGTTGG GAGTGTAAGC ATCGTAAGGT GTTCAGCGGA CCAATACTAA TAAATCGAGG GCTTAACTTT ACAGACCTGT CCAAGAAGCG AAGCGGATTG GGTAACAGGT CGTATGCGAA AACATCCCAA GAATCGAGTC CGAAGGGCGA AGATGATTGG CAGATGTTGA CCGCTAATAA TCTAGAATGT TTCGATACAA TTTTTCTTCT GTATAGTTTT GAGTGGACAT CGTTCATTCA ATAATATCCA GTGACGATAG CTGAGTGGTA CCACCTGTTC CCATACCGAA CACAGTAGTT AAGCACTCAT ACGCCGAAAG TACTTGTCTG GAAACGGGCT GCGAGAATAG GACGTCGCC -3'	50 100 150 200 250 300 350 400
18	Selenomonas	lacticifex	DSM 20757	23S-spacer-5S operon 2	5'-	AAGTGCTGAA AGCATCTAAG CGTGAAGCCT GTCCCGAGAT GAAGTATCTC ATGGAGTAAT CCAGTAAGAT TCCTTGAAGA AGACAAGGTA GATAGGTTGG GAGTGTAAGC ATCGTAAGGT GTTCAGCGGA CCAATACTAA TAAATCGAGG GCTTATCTTA ATAATCTAGA ATGTTTCGAT ACAATTTTTC TTCTGTATAG TTTTGAGTGG ACATGGTTCA TTCAATAATA TCCAGTGACG ATAGCTGAGT GGTACCACCT GTTCCCATAC CGAACACAGT AGTTAAGCAC TCATACGCCG AAAGTACTTG TCTGGAAACG GGCTGCGAAA ATAGGACGCC GCC-3'	50 100 150 200 250 300
19	Zymophilus	raffinosivorans	DSM 20765	23S-spacer-5S	5'-	AAGTGCTGAA AGCATCTAAG CGTGAAACCA GCCTTAAGAT GAGGTTTCTC ACAGAGCAAT CTGGTAAGAC CCCTTGAAGA AGACAAGGTA GATAGGTCGG GAGTGGAAGC GCAGTAATGT GTGCAGCGGA CCGATACTAA TAGGTCGAGG GCTTGACTTA AAGCCAGAAC GAAAACTAAA ATGCGAACAT TTCTTTCTTC TGTATAGTTT TGAGAGAACA AACTCTTAAG ATGGAGTAGT CTGAGGCGAA AGCGGAAGGC AGCGATATCT AAAAAAAGAA TATCTGGTAG TGATAGCCAA GTGGACCCAC CTGTTCCCAT ACCGAACACA GTAGTTAAGC ACTTGAACGT CGAAAGTACT TGGGTGGAAA CGCCCTGCGA AAATAGGACA CCGCC-3'	50 100 150 200 250 300 350

SEQ ID NO	Source			Description	Sequence		
20	Zymophilus	paucivorans	DSM 20759	23S-spacer-5S	5'-	AAGTGCTGAA AGCATCTAAG CGTGAAACCA GCCTTAAGAT GAGGTTTCTC ACAGAGCAAT CTGGTAAGAC CCCTTGAAGA AGACAAGGTA GATAGGTCGG GAGTGGAAGC GCAGTAATGT GTGTAGCGGA CCGATACTAA TAGGTCGAGG GCTTGACTTA AAGCCAGAAC GAATTCTAAA ATGCGAACAT TTCTTTCTTC TGTATAGTTT TGAGAGAACA GACTCTTAAG ATGAGCAGTC TGAGGCGAAA GCTAAAGGCA GCGATATCTA AAAAAAAGAA TATCTGGTAG TGATAGCCAA GTGGACCCAC CTGTTCCCAT ACCGAACACA GTAGTTAAGC ACTTGAACGT CGAAAGTACT TGGGTGGAAA CGCCCTGGGA AAATAGGACA CCGCC-3'	50 100 150 200 250 300 350

Table 2

SEQ ID NO	Description			Sequence	
21	Lactobacillus brevis	Specific probe	5'-	CCAAGTCAACAACGTAGTTGT	-3'
22	Lactobacillus lindneri	Specific probe	5'-	GACACAGGGTTAAATCAAAGTTG	-3'
23	Lactobacillus casei Lactobacillus paracasei ssp. paracasei	Specific probe	5'-	AGGTTTCTGCGACTGCGAAC	-3'
24	Lactobacillus coryniformis ssp.coryniformis Lactobacillus coryniformis ssp.torquens	Specific probe	5'-	ATGTACGTAGTGTTAGTTTAAGGGC	-3'
25	Lactobacillus curvatus	Specific probe	5'-	CTTCTCAGTGCGCAAGCACA	-3'
26	Pediococcus damnosus	Specific probe	5'-	GTGTTCTCAAGAGAAGGATTCG	-3'
27	Pediococcus inopinatus	Specific probe	5'-	GTTCTCAAAGAGAAGATTTCGATATTA	-3'
28	Pectinatus cerevisiiphilus	Specific probe	5'-	TGAGAGCGTAAAACTGCGGACTT	-3'
29	Pectinatus frisingensis	Specific probe	5'-	CAGATAAGTTTCCTGGTTACTG	-3'
30	Pectinatus sp. DSM 20764	Specific probe	5'-	CACTAAGGTGCAGAAAAGAACGT	-3'
31	Megasphaera cerevisiae	Specific probe	5'-	CTTTTCGATGTAGTTGTCAGGATACG	-3'
32	Selenomonas lacticifex	Specific probe	5'-	GTTCATTCAATAATATCCAGTGACG	-3'
33	Zymophilus raffinosivorans	Specific probe	5'-	AACTCTTAAGATGGAGYAGTCTG	-3'
34	Zymophilus paucivorans	Specific probe	5'-	ACTCTTAAGATGAGcAGTCTGA	-3'
35	Pediococcus genus	genus-specific probe	5'-	AGTSTAGTGATACATGGAGCG	-3'
36	Pectinatus genus	genus-specific probe	5'-	GTGAAGTTTTGAGTGTGCAAGA	-3'
37	Megasphaera genus	genus-specific probe	5'-	GACCGAGGACTTGACTTAAGCA	-3'
38	Selenomonas genus	genus-specific probe	5'-	TCCAGTGACGATAGCTGAGT	-3'
39	Zymophilus genus	genus-specific probe	5'-	AAGAATATCTGGTAGTGATAGCCAA	-3'

Table 2 (Cont.)

SEQ ID NO	Description		Sequence	
40	consensus sequence	5'-	GTCGTGAGACAGTTCGGTC	-3'
41	consensus sequence	5'-	CYTAGTACGAGAGGACCGGRR	-3'
42	consensus sequence	5'-	GCTACCCTGGGGATAACAGGC	-3'
43	consensus sequence	5'-	ATCGACGGGGAGGTTTSSCAC	-3'
44	consensus sequence	5'-	CACCTCGATGTCGGCTCRTC	-3'
45	consensus sequence	5'-	CCAAGGGTTGGGCTGTTC	-3'
46	consensus sequence	5'-	AAGGGCCATCRCTCAACGG	-3'
47	consensus sequence	5'-	AAGTGCTGAAAGCATCTAAG	-3'
48	consensus sequence	5'-	TGTGTTCGiiATGGGAACAGGTG	-3'
49	consensus sequence	5'-	TGTGTTCGGAATGGGAACAGGTG	-3'
50	consensus sequence	5'-	TGTGTTCGAAATGGGAACAGGTG	-3'
51	consensus sequence	5'-	TGTGTTCGGTATGGGAACAGGTG	-3'
52	consensus sequence	5'-	TGTGTTCGATATGGGAACAGGTG	-3'
53	consensus sequence	5'-	TGTGTTCGGCATGGGAACAGGTG	-3'
54	consensus sequence	5'-	TGTGTTCGACATGGGAACAGGTG	-3'
55	consensus sequence	5'-	GGCRRYGTCCTAYTYTCSC	-3'
56	consensus sequence	5'-	GGCAGTGTCCTACTTTCCC	-3'
57	consensus sequence	5'-	GGCAGCGTCCTACTTTCGC	-3'
58	consensus sequence	5'-	GGCAGTGTCCTACTTTCGC	-3'
59	consensus sequence	5'-	GGCAGCGTCCTACTTTCCC	-3'
60	consensus sequence	5'-	GYTTMRCTTCYRDGTTCG	-3'
61	consensus sequence	5'-	GCTTAACTTCCGTGTTCG	-3'
62	consensus sequence	5'-	GCTTAACTTCTATGTTCG	-3'
63	consensus sequence	5'-	GCTTAACTTCTGTGTTCG	-3'
64	consensus sequence	5'-	GCTTAACTTCCATGTTCG	-3'
65	consensus sequence	5'-	GCTTAACTTCCGGGTTCG	-3'
66	consensus sequence	5'-	GCTTAACTTCTAGGTTCG	-3'
67	consensus sequence	5'-	GCTTAACTTCTGGGTTCG	-3'
68	consensus sequence	5'-	GCTTAACTTCCAGGTTCG	-3'
69	consensus sequence	5'-	GCTTAACTTCCGAGTTCG	-3'
70	consensus sequence	5'-	GCTTAACTTCTAAGTTCG	-3'

Table 2 (Cont.)

SEQ ID NO	Description				Sequence	
71	consensus sequence			5'-	GCTTAACTTCTGAGTTCG	-3'
72	consensus sequence			5'-	GCTTAACTTCCAAGTTCG	-3'
73	Lactobacillus	brevis	specific probe	5'-	TCGAGAATAATTGAATAATATCTAG	-3'
74	Lactobacillus	brevis	specific probe	5'-	GAGGGAAGAAGTTCTCTTAT	-3'
75	Lactobacillus	lindneri	specific probe	5'-	AACAGAGAAGATATTATCTAGTT	-3'
76	Lactobacillus	lindneri	specific probe	5'-	TTGAGAGAACGAAGTTCGCTCAGGCTTATGAAAAATAAGCAT	-3'
77	Lactobacillus	casei	specific probe	5'-	TTCGTTGGCCGGGTTTGGCCAATGGATTCAGGGTTCTTATGTGG	-3'
78	Lactobacillus	casei	specific probe	5'-	GCGTTTCGATGAAATACACTGGTTCCCGACAACACAAAAACAACAATGA TAGCCAGTT	-3'
79	Lactobacillus Lactobacillus	casei paracasei	specific probe	5'-	TTAGAAACCGGAGCATAAGCGGGCCTGAG	-3'
80	Lactobacillus	paracasei	specific probe	5'-	GCGTGATGGCCGGGCTTTGGCCATTGCGGTCAGGGTCCTTATGTGC	-3'
81	Lactobacillus	paracasei	specific probe	5'-	CAAGTACGTTAAGTTCAAGGCAGCAATTAAACAATGATAGCTAGTT	-3'
82	Lactobacillus	coryniformis	specific probe	5'-	AAAGAAATGAATATCCAGTTTTGAGAGCGCAACGTTCTCAGAAA	-3'
83	Lactobacillus	curvatus	specific probe	5'-	AGGTGCAATGTTAGGCTTTTGAAATGAAATATTACTTATTATGCAGTT	-3'
84	Pediococcus	damnosus	specific probe	5'-	GCCGCGTAAGTGGATCGGAGAA	-3'
85	Pediococcus	inopinatus	specific probe	5'-	GCCGCGGAAGTGGATCGGAGAA	-3'
86	Pediococcus Pediococcus Pediococcus	damnosus inopinatus parvulus	specific probe	5'-	GAGAGAATAAATTTCTTTCACACGA	-3'
87	Pectinatus	cerevisiiphilus	specific probe	5'-	AAAATCATCGAAAAAAATGTTTGGTCTGAGATTTCTTCT	-3'
88	Pectinatus	cerevisiiphilus	specific probe	5'-	CACTCTGGTTGAAGGGCAGGGAACG	-3'
89	Pectinatus	frisingensis	specific probe	5'-	GATTTCATCAAAAAAGAGAAATGTTTGGTCAGAGATTTT	-3'
90	Pectinatus	frisingensis	specific probe	5'-	TATATACCGGCTGAGGTGCTGAGGCACTGAAGG	-3'
91	Pectinatus	sp. DSM 20764	specific probe	5'-	AATTTCATCTATAAATGTTTGGTCCTGATTTCTTCT	-3'
92	Pectinatus	sp. DSM 20764	specific probe	5'-	AGATTAGTTCCTGGTTTACTTTATATATGAGCACTAAGGTGCAGAAAAG AACGT	-3'
93	Pectinatus	sp. DSM 20764	specific probe	5'-	AGGAAACGCGGCGTTCGTAA	-3'
94	Selenomonas	lacticifex	specific probe	5'-	TAATAATCTAGAATGTTTCGATACAATTTTTCTTCTGTATAGTTTTGAG TGGACAT	-3'
95	Zymophilus	raffinosivorans	specific probe	5'-	GAGGCGAAAGCGGAAGGCAGCGAT	-3'
96	Zymophilus	paucivorans	specific probe	5'-	GAGGCGAAAGCTAAAGGCAGCGAT	-3'
97	Megasphaera	cerevisiae	specific probe	5'-	AATCCTGAAACGAATTCAGTGGTGATGGCTGCAGGGA	-3'

Table 2 (Cont.)

SEQ ID NO	Description				Sequence	
98	Detection of all *Lactobacillaceae* relevant to brewing for differentiation from other bacteria relevant to brewing			5'-	TATGGAAGTAAGACCCCTGA	-3'
99				5'-	AGATGATCAGGTAGATAGGCT	-3'
100				5'-	AGATGATCAGGTCGATAGGTT	-3'
101				5'-	AGATGATCAGGTAGATAGGTT	-3'
102				5'-	TACTAATCGGTCGAGGACTTAACCA	-3'
103				5'-	ATACTAATCAGTCGAGGACTTAACCA	-3'
104	Pectinatus	genus	genus-specific probe	5'-	GAAGCGGACTGGTACTAATAAGCCGAGAGCTT	-3'
105	Selenomonas	genus	genus-specific probe	5'-	CAGCGGACCAATACTAATAAATCGAGGGCTTA	-3'
106	Zymophilus	genus	genus-specific probe	5'-	AGCGGACCGATACTAATAGGTCGAGGGCTTGACTTAAA	-3'
107	Megasphaera	genus	genus-specific probe	5'-	GGAGCGGACCGGTACTAATAGACCGAGGACTT	-3'

Table 3

	SEQ ID NO 21	SEQ ID NO 22	SEQ ID NO 23	SEQ ID NO 24	SEQ ID NO 25	SEQ ID NO 26	SEQ ID NO 27	SEQ ID NO 28	SEQ ID NO 29	SEQ ID NO 30	SEQ ID NO 31	SEQ ID NO 32
Lactobacillus brevis	+	-	-	-	-	-	-	-	-	-	-	-
Lactobacillus lindneri	-	+	-	-	-	-	-	-	-	-	-	-
Lactobacillus casei	-	-	+	-	-	-	-	-	-	-	-	-
Lactobacillus paracasei paracasei	-	-	+	-	-	-	-	-	-	-	-	-
Lactobacillus coryniformis coryniformis	-	-	-	+	-	-	-	-	-	-	-	-
Lactobacillus coryniformis torquens	-	-	-	+	-	-	-	-	-	-	-	-
Lactobacillus curvatus	-	-	-	-	+	-	-	-	-	-	-	-
Pediococcus damnosus	-	-	-	-	-	+	-	-	-	-	-	-
Pediococcus inopinatus	-	-	-	-	-	-	+	-	-	-	-	-
Pectinatus cerevisiiphilus	-	-	-	-	-	-	-	+	-	-	-	-
Pectinatus frisingensis	-	-	-	-	-	-	-	-	+	-	-	-
Pectinatus sp. DSM 20462	-	-	-	-	-	-	-	-	-	+	-	-
Megasphaera cerevisiae	-	-	-	-	-	-	-	-	-	-	+	-
Selenomonas lacticifex	-	-	-	-	-	-	-	-	-	-	-	+
Zymophilus raffinosivorans	-	-	-	-	-	-	-	-	-	-	-	-
Zymophilus paucivorans	-	-	-	-	-	-	-	-	-	-	-	-

	SEQ ID NO 33	SEQ ID NO 34	SEQ ID NO 35	SEQ ID NO 36	SEQ ID NO 37	SEQ ID NO 38	SEQ ID NO 39	SEQ ID NO 40-45
Lactobacillus brevis	-	-	-	-	-	-	-	+
Lactobacillus lindneri	-	-	-	-	-	-	-	+
Lactobacillus casei	-	-	-	-	-	-	-	+
Lactobacillus paracasei paracasei	-	-	-	-	-	-	-	+
Lactobacillus coryniformis coryniformis	-	-	-	-	-	-	-	+
Lactobacillus coryniformis torquens	-	-	-	-	-	-	-	+
Lactobacillus curvatus	-	-	-	-	-	-	-	+
Pediococcus damnosus	-	-	+	-	-	-	-	+
Pediococcus inopinatus	-	-	+	-	-	-	-	+
Pectinatus cerevisiiphilus	-	-	-	+	-	-	-	+
Pectinatus frisingensis	-	-	-	+	-	-	-	+
Pectinatus sp. DSM 20462	-	-	-	+	-	-	-	+
Megasphaera cerevisiae	-	-	-	-	+	-	-	+
Selenomonas lacticifex	-	-	-	-	-	+	-	+
Zymophilus raffinosivorans	+	-	-	-	-	-	+	+
Zymophilus paucivorans	-	+	-	-	-	-	+	+